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Increased (Antibiotic-Resistant) Pathogen Indicator Organism Removal during (Hyper-)Thermophilic Anaerobic Digestion of Concentrated Black Water for Safe Nutrient Recovery

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Abstract: Source separated toilet water is a valuable resource for energy and fertilizers as it has a high concentration of organics and nutrients, which can be reused in agriculture. Recovery of nutrients such as nitrogen, phosphorous, and potassium (NPK) decreases the dependency on energy-intensive processes or processes that rely on depleting natural resources. In new sanitation systems, concentrated black water (BW) is obtained by source-separated collection of toilet water. BW-derived products are often associated with safety issues, amongst which pathogens and antibiotic-resistant pathogens. This study presents results showing that thermophilic (55–60 °C) and hyperthermophilic (70 °C) anaerobic treatments had higher (antibiotic-resistant) culturable pathogen indicators removal than mesophilic anaerobic treatment. Hyperthermophilic and thermophilic anaerobic treatment successfully removed *Escherichia coli* and extended-spectrum β-lactamases producing *E. coli* from source-separated vacuum collected BW at retention times of 6–11 days and reached significantly higher removal rates than mesophilic (35 °C) anaerobic treatment ($p < 0.05$). The difference between thermophilic and hyperthermophilic treatment was insignificant, which justifies operation at 55 °C rather than 70 °C. This study is the first to quantify (antibiotic-resistant) *E. coli* in concentrated BW (10–40 gCOD/L) and to show that both thermophilic and hyperthermophilic anaerobic treatment can adequately remove these pathogen indicators.

Keywords: source separation; black water; nutrient recovery; (hyper-)thermophilic anaerobic digestion; pathogen removal; antibiotics resistance

1. Introduction

The growing world population causes an increased demand for fertilizers to provide enough food. Currently, the agro-industry is dependent on artificial fertilizers for food production. Natural fertilizer resources for phosphorous (P) [1,2] and potassium (K) [3,4] are becoming scarce, whereas nitrogen (N) is harvested through the energy-intensive Haber–Bosch process [5]. Nutrients from human excreta, which are currently wasted in certain regions, form a good source for alternative fertilizer products. When recovered in a hygienic way, nutrients from human excreta can contribute to a more

circular agro-food chain and to a decreased dependency on depleting artificial fertilizer sources and energy-intensive processes. For instance, 25%, 45%, and 59% of the N, P, and K applied with synthetic fertilizers in agriculture in The Netherlands can be potentially replaced by nutrients from human excreta [6].

Anaerobic digestion (AD) is a widely applied technology for treatment and nutrient recovery from waste streams. Efficient nutrient recovery from domestic wastewater requires novel sanitation approaches. New sanitation focuses on the different properties, such as composition and concentration, of each domestic waste stream [6]. Treatment can thus be more tailored to recover resources when these domestic waste streams are separated at the source. The stream containing only toilet water, which is called black water (BW), is a valuable stream with organics and nutrients [6]. When BW is collected separately, preferably by vacuum toilets to minimize the dilution of valuable compounds by flushing water, it is high in concentration [6,7] and low in external heavy-metals deriving from for instance industrial waste streams [8], therefore suited for energy and nutrient recovery.

Separate collection of BW through vacuum toilets is already applied in multiple locations in The Netherlands [9,10] and other countries around the world [11,12], however, recovered nutrients from human excreta are currently not considered a suitable source to use in fertilizers due to presumed safety issues [13]. Even healthy people excrete pathogenic bacteria that can cause a health impact for other people [14]. When human excreta-based compounds are applied on a field, pathogens can re-enter the food chain through crops [15]. Furthermore, increased human and animal intake of antibiotics causes the emergence of antibiotic-resistant bacteria (ARB), due to an increased selective pressure for bacteria that can resist antibiotics [16]. These ARB are of great concern as they are hard to treat with antibiotics when they cause an infection [17].

Antibiotics usage is a delicate dilemma. On one hand, it leads, amongst many other benefits, to significantly increased human life expectancy [18]. Antibiotics are widely applied to treat infections in humans, as well as to treat or prevent diseases and increase growth rates in livestock and aquacultures [19–23]. On the other hand, excessive antibiotics usage has resulted in the emergence of resistant bacteria causing hardly treatable diseases. Both the World Economic Forum [24] and the World Health Organization [25] have described antibiotic resistance as one of the major threats to humanity in the coming century. Antibiotics or their metabolites enter wastewater treatment plants (WWTPs) and subsequently end up in surface waters through the sewage system, since WWTPs were not designed to remove antibiotics [22,26–28]. Not only surface waters, but soils and sediments also suffer from the accumulation of antibiotics due to the usage of veterinary antibiotics and subsequent application of contaminated manure to the soil [20–22,27,29,30]. In the intestines of living organisms but also in surface waters, WWTPs, and soils, the presence of antibiotics (residues) create a selective environment for the emergence of ARB [16,17,20,28,31–33]. For instance, Sengeløv et al. found tetracycline-resistant bacteria increase after spreading pig manure onto the land [34]. Also, it was already shown that antibiotic resistant indicator organisms are present in black water, and thus the relevance of antibiotic resistance removal during black water AD before resource reuse in agriculture was demonstrated [35].

ARB possess specific antibiotic resistance genes (ARGs) providing them with resistance for antibiotics [20]. These ARGs can spread through vertical gene transfer during replication of the bacterium, but also via horizontal gene transfer (HGT). During HGT, mobile genetic elements (MGEs), such as plasmids, integrons, transposons, and gene cassettes, are transported between bacteria [20,28]. HGT then imposes the risk of the transfer of an ARG from a harmless bacterium to a human pathogen [23,32,36]. The efficacy of antibiotic treatments is reduced when an MGE is transferred to a human pathogen [34,36].

Exposure to high temperatures is known to be the most effective method to inactivate pathogens [37]. Kjerstadius et al. found hygienization of sludge, in terms of *Salmonella* and *Escherichia coli (E. coli)* removal during anaerobic digestion at 55 and 60 °C in 20 L continuous stirred tank reactors (CSTRs) (hydraulic retention time (HRT) = seven days) [38]. Bendixen already showed that thermophilic treatment (50–55 °C) of manure and slurry decreases pathogen survival compared to mesophilic treatment [39].

Other studies also confirm that (hyper-)thermophilic anaerobic treatment is more efficient in pathogen removal than mesophilic treatment [40–42]. Also, ARG removal is thought to increase at higher temperatures. For instance, Diehl and LaPara found increasing removal of ARG coding for tetracycline resistance at increasing temperatures in the range of 22 to 55 °C [18]. However, the effect of temperature on the inactivation of (antibiotic-resistant) pathogens or indicator organisms was never studied during concentrated BW treatment. In this study, *E. coli* a thermotolerant Gram-negative bacterium, was selected as indicator for pathogens and antibiotic resistant pathogens. The (hyper-)thermophilic anaerobic treatment technologies are developed to remove ARB and ARG from black water to guarantee safe reuse of human excreta-based nutrients and organics as fertilizer. To the best of our knowledge, no studies have been performed on the effect of temperature on the ARB or indicator organisms' removal during anaerobic (concentrated) BW treatment. In this study, different operational anaerobic systems that are treating actual vacuum-collected BW in upflow anaerobic sludge blanket (UASB) reactors at 35, 55, 60, and 70 °C are compared to see if and to what extent (antibiotic-resistant) indicator organisms are removed.

2. Materials and Methods

2.1. Sampling

We selected three test sites for sampling BW and effluent after treatment in a UASB. All test sites apply on-site source separated sanitation with vacuum toilets and thus produce similar BW streams. The BW is treated in similar UASB reactors at each sampling site, so the effect of temperature, which is different in each test site, can be determined. Only Noorderhoek, the mesophilic reference site, is slightly different in BW composition due to co-collection of kitchen waste (KW) and slightly different vacuum toilets. An overview of all relevant information of the sampling sites is given in Table 1. The average influent concentrations and the reactor performance can be found in Table S1.

Table 1. Overview of operational parameters and user info of the selected sampling sites (Noorderhoek, DeSaH, and WUR).

	Sampling	**Noorderhoek**	**DeSaH**	**WUR**
	Site	**Sneek**	**Sneek**	**Wageningen**
Toilet system		Regular vacuum toilets (+ kitchen grinders)	Male ultra-low flush volume vacuum toilets	Male and female ultra-low flush volume vacuum toilets
Urinal alternative		No	Yes	Yes
Average flush volume	L	1–1.5	0.7	0.2–0.8
Temperature(s)	°C	35	60	55 and 70
Hydraulic retention time (HRT)	Days	11	11	6–8
Reactor volume	L	40,000	500	4.9
Sample period		17 July 2018–11 July 2019	29 April 2019–11 July 2019	16 May 2019–04 September 2019
Samples	n	9	5	8
Users		232 households, 192 family homes, 40 apartments for elderly people (nursing homes). A total of 330 residents.	Office building, 60 male employees	Working area, 80 people, mainly students and PhD students in the age of 20–35
Storage of black water (BW) prior to treatment		The BW and kitchen waste (KW) were stored for a brief period (a couple of hours) in an underground buffer vacuum tank.	The BW was stored in a well-mixed buffer tank for approximately 1.5 to 3 days at room temperature.	After collection, the BW was stored at room temperature in a mixed tank (V = 200 L) with an estimated retention time of 7 days prior to treatment in the reactors.

The first test site at Environmental Technology of Wageningen University and Research (WUR) has two vacuum toilets collecting BW from roughly 80 employees with small and big flush volumes of 0.2 and 0.8 L, respectively. The BW was treated in two separately operated laboratory-scale UASB reactors at 55 and 70 °C. The second sampling site is situated at the office of DeSaH B.V, Sneek, The Netherlands. The DeSaH UASB reactor (DeSaH) operated under thermophilic conditions with black water from eight ultra-low flush volume vacuum toilets. Both at WUR and DeSaH, male urinals were diverted from the BW. However, at WUR the urine fraction of the collected BW is bigger due to the presence of a female ultra-low flush vacuum toilet. The third sampling site is the demonstration site Noorderhoek in Sneek, The Netherlands (Noorderhoek) [9]. In Noorderhoek BW and kitchen waste (KW) is treated from the surrounding neighborhood of 232 houses. These houses are equipped with normal vacuum toilets (1–1.5 L per flush) and kitchen grinders. This BW and KW were treated under mesophilic conditions at the Noorderhoek WWTP.

Samples from all reactors (0.1–0.2 L) (9, 5, and 8 at Noorderhoek, DeSaH, and WUR, respectively) were grabbed from the influent pipe. Effluent samples (0.1–0.2 L) were grabbed directly from the effluent pipe of all reactors. All samples were blended and kept cool during transport and were analyzed for (antibiotic-resistant) bacteria at Wetsus, Leeuwarden, The Netherlands within 24 h.

2.2. Analytical Methods

To quantify the removal of (antibiotic resistant) bacteria, *E. coli* was selected as an indicator for fecal contamination because of its prevalence in the human gut, thermotolerant properties, and easy detection method [43]. Plating assays were preferred as analysis method, since preliminary quantitative polymerase chain reaction (qPCR) analyses of 16S rRNA and ARGs presumably showed low removal due to non-viable bacteria amplification (data shown in Section S3). Also in multiple other recent studies with *E. coli* as indicator organism for similar waste streams, plating assays have been applied as quantification method [35,44,45]. The colony-forming units (CFUs) were determined according to the ISO 8199 standard method. Tryptone Bile X-glucuronide (TBX) agar (EWC diagnostics, Steenwijk, The Netherlands; T703.02) which contains chromogenic substrates that allow for easy identification based on glucuronidase activity was selected to quantify *E. coli* [46]. Colonies suspected of *E. coli* were counted based on morphology and color as indicated by the manufacturer of the agar plates. For antibiotic resistance, ChromID ESBL agar (bioMérieux, Marcy l'Etoile, France; 43481) plates were used, because they select for extended-spectrum β-lactamases (ESBL), which are produced by β-lactam resistant bacteria, which can be found in human feces [47,48]. Carbapenems are usually the next treatment option, and therefore carbapenem-resistant *Enterobacteriaceae* are of big concern due to the limited treatment options [47]. ChromID CARBA (bioMérieux, Marcy l'Etoile, France; 43861) plates were therefore selected to quantify carbapenem-resistant *E. coli.* Dilution steps were performed until the sample CFU concentration was between 0 and 100 CFUs/mL. Samples were diluted in physiological salt (BioTrading; K110B009AA) and filtered using sterile 0.45 µm filters (Merck, Darmstadt, Germany; EZHAWG474). The filters were placed on agar plates and incubated at 37 °C, since the bacteria that were to be quantified are mesophilic species, for 24 h or at 37 °C for 4 h and then at 44 °C for 18–24 h to prevent background growth.

2.3. Statistical Methods

RStudio v1.1463 was used to perform the statistical analysis on the removal of *E. coli* and β-lactamase-producing *E. coli* (CARBA plates were not incorporated in the statistical tests since there were almost no CFUs detected on any of these plates). The script can be found in Section S2. Shapiro–Wilk tests were performed to determine the normality of all data sets. Paired *t*-tests and Wilcoxon signed-rank tests assuming unequal variance where performed to test removal for each reactor with normally and non-normally distributed data, respectively. The mean removal of all sample dates for each plate type at the different temperatures was compared in an unpaired *t*-test or unpaired

Wilcoxon rank sum test. A confidence interval of 95% was selected to reject the null hypothesis for all statistical tests.

3. Results and Discussion

In this study, we investigated the removal of *E. coli*, β-lactamase-producing *E. coli* and carbapenemase-producing *E. coli* at 35, 55, 60, and 70 °C. Carbapenemase-producing *E. coli* were detected only in two influent samples from Noorderhoek (35 °C), average data is shown in Table S5. In general, the influent bacteria concentrations at DeSaH and Noorderhoek were higher than those observed at WUR. The bacteria concentrations at DeSaH are higher, because dilution of fecal matter by urine and connected flush water occurs to a lesser extent compared to WUR since male urinals and female toilets are diverted from the BW stream. Also, water usage by flushing events was lower at DeSaH. At Noorderhoek bacterial concentrations are higher than at WUR because this is a populated community area including an apartment block for elderly people. As such, the fraction of antibiotics usage is probably higher than in the office buildings of DeSaH and WUR, resulting in higher ARB concentrations. Additionally, it was found that opportunistic bacteria such as *Enterobacteria* are more abundant in the gut microbiome of elderly people [49].

3.1. Removal at Each Temperature

In the influent of Noorderhoek and DeSaH, the *E. coli* CFUs (Figure 1) were present in high concentrations (log 5.6–6.0), which is in line with results obtained with vacuum collected BW in previous studies [35]. At DeSaH (60 °C) *E. coli* colonies were not detected in the effluent, whereas at Noorderhoek (35 °C) *E. coli* and β-lactamase-producing *E. coli* were not completely removed (2.7 log reduction). At 35 °C (Noorderhoek), for both plate types the CFU concentrations in the effluent were significantly lower than in the influent ($p < 0.05$), but removal was not complete. On the other hand, at 60 °C *E. coli* were completely removed in all samples. The same was observed for the 60 °C ESBL plates (Figure 2).

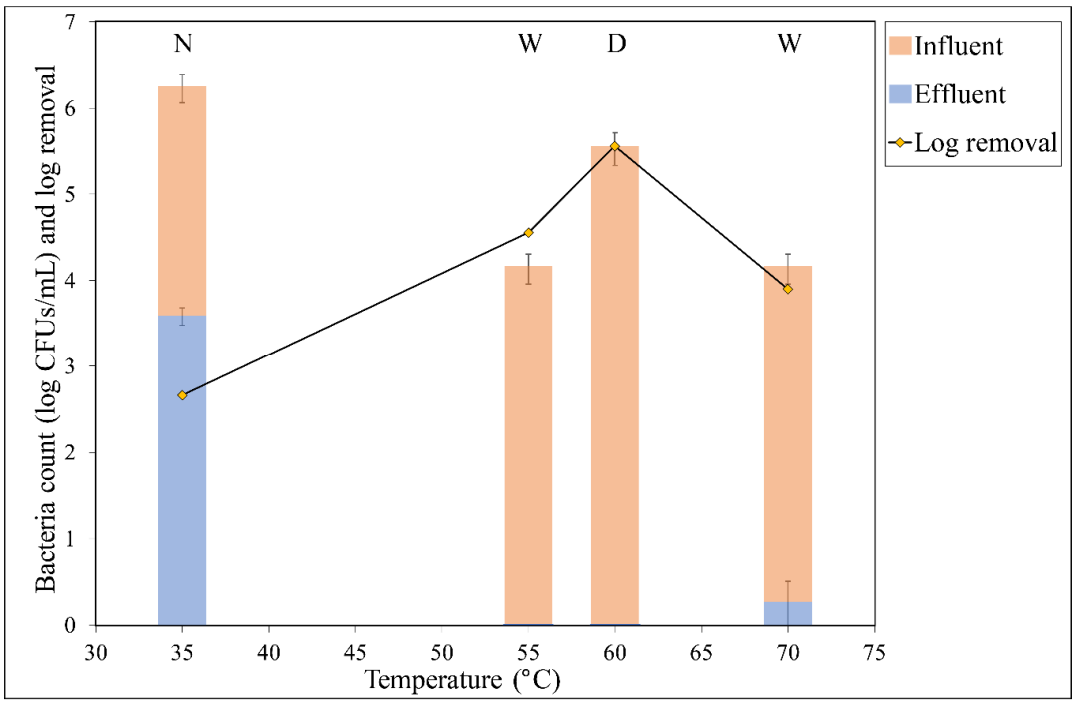

Figure 1. Mean colony concentrations for Tryptone Bile X-glucuronide (TBX) plates selecting for *E. coli* with influent and effluent samples from upflow anaerobic sludge blanket (UASB) reactors (Noorderhoek, DeSaH, and WUR, indicated in the figure as N, D, W, respectively) treating concentrated black water (BW) at 35, 60, and 55 or 70 °C, respectively. A flat blue line indicates that the mean colony concentration was below the detection limit or too low to form a visible bar. Influent and effluent data is plotted on a logarithmic scale with base 10. The black line shows the total log removal.

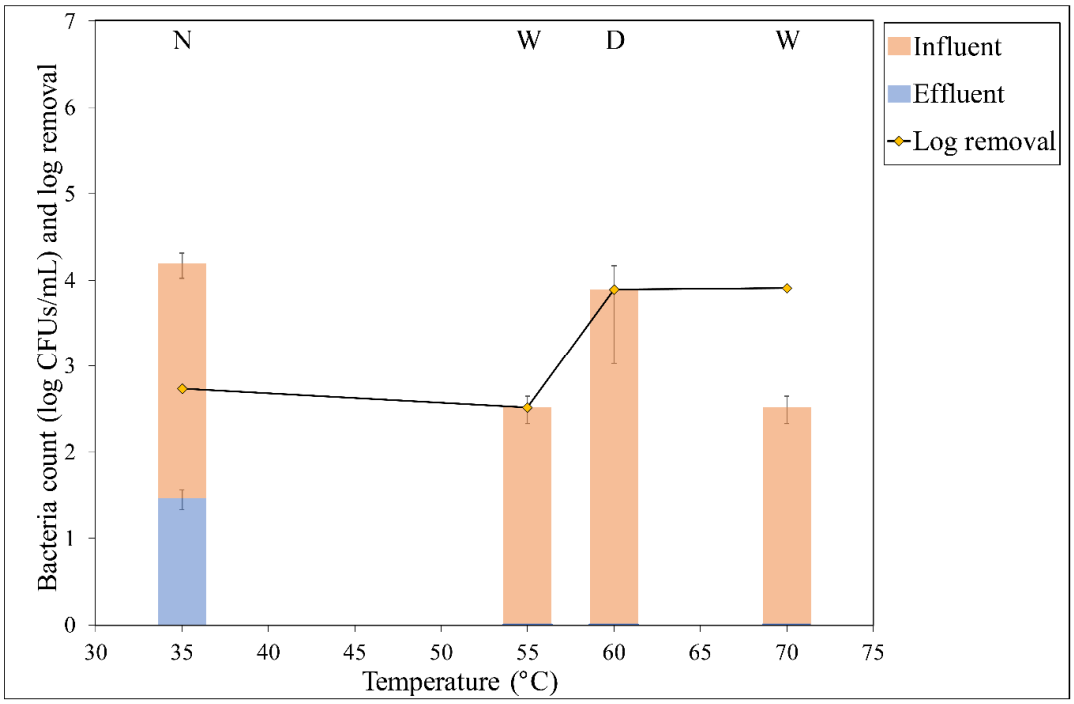

Figure 2. Mean colony concentrations for extended-spectrum β-lactamase (ESBL) plates selecting for extended spectrum β-lactamase-producing *E. coli* with influent and effluent samples from upflow anaerobic sludge blanket (UASB) reactors (Noorderhoek, DeSaH, and WUR, indicated in the figure as N, D, W, respectively) treating concentrated black water (BW) at 35, 60, and 55 or 70 °C, respectively. A flat blue line indicates that the mean colony concentration was below the detection limit or too low to form a visible bar. The log removal at 70 °C is higher than at 55 °C despite the same influent concentration, due to a mean effluent concentration of <1 CFU/mL in the 70 °C effluent, which results in a negative exponent. Influent and effluent data is plotted on a logarithmic scale with base 10. The black line shows the total log removal.

The initial 4.2 log *E. coli* concentration at the WUR demonstration site influent was almost completely removed at both temperatures (55 and 70 °C). Also, ESBL plates showed (almost) complete removal at both temperatures. The effluent ESBL concentration at 55 °C and 70 °C was 0 and 0.04 CFUs/mL, respectively. Overall, both TBX and ESBL plates showed significant removal at both 55 and 70 °C ($p < 0.05$).

3.2. Effect of Temperature

The removal of *E. coli* and β-lactamase-producing *E. coli* was significantly lower ($p < 0.05$) at mesophilic conditions (Noorderhoek) compared to thermophilic conditions (WUR, 55 °C) and hyperthermophilic conditions (WUR, 70 °C). Noorderhoek was the only sampling site were β-lactamase-producing *E. coli* in the effluent exceeded a concentration of 1 CFU/mL. The difference in removal at 35 °C and 60 °C is insignificant due to the smaller sample size at 60 °C, however Figures 1 and 2 show that both *E. coli* and β-lactamase-producing *E. coli* were completely removed at 60 °C. This shows that all (hyper-)thermophilic treatments adequately remove *E. coli* and β-lactamase-producing *E. coli*, whereas at mesophilic conditions the removal is incomplete.

We showed that thermophilic and hyperthermophilic treatment removes *E. coli* and β-lactamase-producing *E. coli* to a higher extent than mesophilic treatment. There was no significant difference between β-lactamase-producing *E. coli* and *E. coli* removal at 55, 60, and 70 °C ($p > 0.05$). This shows that concerning the removal of the aforementioned *E. coli* species, treatment in a continuous system

operated at a temperature of at least 55 °C and an HRT of at least six days is sufficient to remove *E. coli* indicator organisms.

Similarly, Beneragama et al. found 100% and 90% multi-drug resistant bacteria removal with thermophilic anaerobic digestion (55 °C) and mesophilic anaerobic digestion (35 °C), respectively, during treatment of dairy manure and co-digestion of dairy manure and waste milk in a 22-day batch experiment [50]. In dairy manure, Pandey and Soupir found increased *E. coli* indicator organism removal at 52.5 °C as compared to mesophilic conditions [51]. The current study showed that pathogen indicator organisms were adequately removed at thermophilic conditions and that the studied antibiotic-resistant indicator organisms have no increased thermotolerance compared to organisms that are susceptible to antibiotics. In practice, removal of antibiotic-resistant pathogens could mobilize ARGs which could transpose to pathogens during treatment or during application to the soil as was earlier demonstrated in manure amended soils [52]. However, several studies with manure and primary/secondary WWTP sludge already indicated that also ARG removal is increased at thermophilic conditions [33,53–55]. For instance, Jang et al. found increased ARG removal at thermophilic conditions (55 °C) as compared to mesophilic conditions (35 °C) during anaerobic sludge digestion in batch during a period of 35 days [56]. Burch et al. found that there was no further increase in ARG removal when the temperature exceeded 55 °C, which is in line with the results of pathogen indicator organism removal obtained in this paper [57]. In an earlier study, the same authors found that an increase in temperature from 22 to 55 °C does improve the removal of the same ARGs [18]. Furthermore, it is shown that thermophilic AD blocks HGT pathways by inactivating MGEs, thus decreasing the mobility of ARGs, and by decreasing the amount of potential hosts [33,55]. The risk of HGT during application of BW-based fertilizers should be further researched, but the combined effect of increased ARG removal and gene immobilization during thermophilic AD leads to the hypothesis that thermophilic AD of BW is also safe with regards to HGT. Although additional research is needed, our results of pathogen indicator organism removal and results regarding HGT at thermophilic temperatures as described above indicates that fertilizer products from thermophilic anaerobic digestion of BW can be safely applied.

3.3. Next Steps in Process Optimization

For mesophilic treatment of BW in an UASB, HRTs of eight and four days are recommended at temperatures of 25 and 35 °C respectively [7,9]. Therefore, a further increase of temperature to (hyper-)thermophilic conditions should in principle allow for much shorter HRTs than the 6–11 days in this study [58]. Treatment at 70 °C may give more certainties regarding pathogen removal. However, anaerobic digestion at 70 °C is more difficult to operate because of decreased methanogenic activity and increased ammonia inhibition [58,59]. This study showed that treatment at 55 °C was sufficient for *E. coli* removal. The complicated AD at 70 °C along with the results in this study regarding *E. coli* removal at 55 °C advocate a thermophilic AD for hygienization of BW preferably at lower HRTs (two to four days). A shorter HRT is not expected to compromise pathogen removal, since an exposure time of 2 h at 55 °C was found to be sufficient to reach EU limits regarding *E. coli*, but also *Salmonella* reduction for usage of sewage sludge in agriculture [38,60]. In our study only UASB reactors, in which the HRT is uncoupled from the sludge retention time (SRT) [61], were assessed. Removal of pathogens can also be associated with filtration and sedimentation [62], so the SRT could be an additional factor in the pathogen removal. The effect of HRT and SRT on pathogen removal should be investigated in more detail. Ammonia (NH_3) is one of the other factors which stimulates pathogen removal, especially in BW treatment [63,64]. In this study the NH_3 concentrations, roughly 390 mg NH_3-N/L, were lower than the threshold for pathogen inactivation of 560 mg NH_3-N/L [63]. Besides only *E. coli* and antibiotic-resistant *E. coli*, thus mainly pathogen indicators, were quantified. This paper shows high *E. coli* indicator organism removal which indicates a good potential for safe nutrient recovery with thermophilic AD, so follow-up research should focus on the removal of a broader range of (antibiotic-resistant) pathogens, like enteric viruses, helminth eggs, and *Salmonella* as well as spore-forming and thermotolerant bacteria

such as *Clostridia* [38,65]. In addition to pathogen indicator organism and ARG removal, more research is required to study antibiotics removal. As antibiotics are the driving factor behind the emergence of antibiotic resistance, and research by others shows contradictory results, e.g., Feng et al. found that thermophilic anaerobic digestion removes the majority of the antibiotics, but some persist in the effluent [66].

Temperature is just one of the possible steps to achieve pathogen removal. Although this study already showed almost complete removal of pathogen indicator organisms at (hyper-)thermophilic conditions, further work should focus on optimizing process parameters such as HRT, SRT, and ammonia toxicity. Additionally, the indicator organisms and the pathogenic organisms mentioned above should be monitored during treatment and after field application with (qPCR) quantification methods. Furthermore, the fate of other pollutants (e.g., micropollutants and antibiotics) should be assessed as well to guarantee recovery of hygienically safe nutrients.

4. Conclusions

The aim of this study was to assess the pathogen removal during (hyper-)thermophilic AD of concentrated BW at laboratory- and pilot-scale. Both thermophilic (55–60 °C) and hyper-thermophilic (70 °C) AD removed pathogen (indicator) organisms (*E. coli* and antibiotic-resistant *E. coli*) to levels below or close to the detection limit. At mesophilic (35) conditions only an incomplete removal (2.7 log reduction) of aforementioned pathogen indicator organisms was achieved. Therefore, we propose thermophilic AD for treatment of (concentrated) BW to recover hygienically safe fertilizer products. Further work should focus on the optimization of other process conditions (HRT, SRT, ammonia toxicity) for high rate thermophilic treatment of BW as well as on the analysis of a broader range of pathogens.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/22/9336/s1, Section S2: R script used for summarizing data and performing statistical analysis, Table S1: Average influent properties and reactor performance, Table S2: Characteristics of primers used for qPCR analysis and accession number (EMBL) of each target amplicon, Table S3: Average of counted colony formatting units (CFU) per mL of sample filtered in 0.45 µm membranes for both reactors (influent and effluent), Table S4: Log concentration of ARG genes (*qnrS* and *sul1*) as well as bacteria (16S) from the DNA extracts of both UASB reactors. Data is shown in log copy/ng DNA, Table S5: Average CFU concentrations per sampling site and plate type, Table S6: *p* values for paired *t*-tests or Wilcoxon signed rank tests testing significance of difference between influent and effluent bacteria concentrations. TBX and ESBL stand for *E. coli* and extended-spectrum β-lactam producing *E. coli*, Table S7: *p*-values for unpaired *t*-tests or Wilcoxon rank sum tests testing significance of the difference between the removal of bacteria at different temperatures. TBX and ESBL stand for *E. coli* and extended-spectrum β-lactam producing *E. coli.* The numbers that follow indicate the two temperatures that are compared.

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